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ORIGINAL ARTICLE

Relative matrix effects: A step forward using standard line slopes and ANOVA analysis

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Method validation

Abstract One of the alternative methods to identify and study the matrix effect is by determination of "relative" matrix effect. In this experiment % coefficient of variance of standard line slopes are calculated. First, six standard lines are prepared from single plasma lot. In another experiment standard line slopes are compared from six different lots of plasma. All these standard curves are prepared by using three different types of IS (internal standard). From all these experiments it is observed that using SIL-IS (stable isotope labeled-internal standard) is one of the best approach in methods having matrix effects. Alternatively, analog IS is a cost effective approach. After comparing a large number of calibration curve slopes, it can be recommended that during every bioanalytical method validation, where the sample size is >50, scientist should perform the "relative" matrix effect experiment by standard line slope method. In selected cases, the precision of standard line slopes in six different lots of a biofluid was compared with precision values determined six times in a single lot. The results of these studies indicated that the variability of standard line slopes in different lots of a biofluid [precision of standard line slopes expressed as coefficient of variation, CV (%)] may serve as a good indicator of a relative matrix effect and, it is suggested, this precision value should not exceed 5% for the method to be considered reliable and free from the relative matrix effect liability.

1. Introduction

Now-a-days, pharmaceutical companies are developing dosage forms where concentration ranges from pictogram to famtogram. These innovations demand the applied analytical methods to be highly sensitive, so that they can quantify the lowest concentration level available during pharmacokinetic studies. To achieve the lowest concentration, scientists are shifting their detection technique from conventional ultraviolet–visible spectrometry (UV–vis) or fluorescence detection to more sensitive mass-spectrometry (MS) detection. Bio-analytical scientists face many challenges during development and routine analysis of matrix samples (i.e., plasma, blood etc.). One of the critical challenges is matrix effect (ME) (Smeraglia et al., 2002; Annesley, 2003; Fu et al., 1998; Jemal et al., 2010; Du and White, 2008; Steene and Lambert, 2008; Erin et al., 2007). ME is the effect of unwanted ions

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generation during the detection of ions of analyte of interest. Mostly, those undesired ions come from the matrix itself i.e. from plasma/blood/urine etc., or the salt present in the analyte or in matrix, or different phospholipids present in the sample matrix, or any degradation products formed inside the matrix, or interference from any metabolic products etc. So, for any bio-analytical scientist one of the prime challenges is to overcome the matrix effect related issues.

ME is defined as the degree of ion suppression or ion enhancement during liquid chromatography–mass spectrometry (LC–MS) analysis. Matrix effect can be determined qualitatively as well as quantitatively. For qualitative analysis post column infusion techniques are the most popular approach. Matrix factor (MF) calculation, calibration curve slope determination, calculation of accuracy of lowest concentration samples etc. are some approaches for quantitative determination of ME. MF is the ratio of analyte response in post extracted plasma sample to the analyte response in neat standard solution. MF > 1, signifies ion enhancement, MF < 1 signifies ion suppression and MF = 1 implies that analytical method is free from matrix effect.

Here, ''relative'' and ''absolute'' matrix effects were studied. ''Relative'' matrix effect is defined as the comparative studies of various matrix effect determining parameters like, standard line slope, matrix factor etc., among different lots of same matrices (i.e. plasma-to-plasma, urine-to-urine etc.). On the other hand, when these parameters are compared using a single lot of matrix, it is termed as ''absolute'' matrix effect.

Regarding causality of MEs, there are a few that have been reported till date. Among them, the prominent causes are as follows: first one is the chromatographic conditions (Erin et al., 2007; Gosetti et al., 2010; Bennett and Hairui, 2004.), which includes improper gradient elution, improper selection of column and mobile phase etc. This can be minimized by different column chemistries, changing the gradient elution and mobile phase with fewer additives is a better option. One of the critical challenges is matrix effect (ME) (Smeraglia et al., 2002; Annesley, 2003, 2007; Fu et al., 1998; Jemal et al., 2010; Du and White, 2008; Steene and Lambert, 2008; Erin et al., 2007; Gosetti et al., 2010; Bennett and Hairui, 2004; Omnia et al., 2007, 2008; Mei et al., 2003; Mallet et al., 2004; Matuszewski et al., 2003; Dams et al., 2003). Secondly, applied ionization technique, as it is reported that ESI is more prone to MEs than the APCI (Smeraglia et al., 2002; Annesley, 2003; Erin et al., 2007; Gosetti et al., 2010; Omnia et al., 2008; Mei and Korfmacher, 2005; Liang et al., 2003; Souverain et al., 2004; Ghosh et al., 2010; Buhrman et al., 1996). This is mainly because of the different ion formation mechanism in both the techniques. Thirdly, ionization polarity, where positive polarity showed more MEs in comparison to negative polarity (Omnia et al., 2008; Pucci et al., 2009.) and perhaps the most important source of ME is the inefficient extraction technique used to extract the analyte and/or metabolite from the specified matrix (Smeraglia et al., 2002; Annesley, 2003; Fu et al., 1998; Jemal et al., 2010; Erin et al., 2007; Gosetti et al., 2010; Omnia et al., 2008; Mei et al., 2003; Mallet et al., 2004; Matuszewski et al., 2003; Dams et al., 2003; Mei and Korfmacher, 2005; Shena et al., 2005; Chin et al., 2004; Avery, 2003; Marchese et al., 1998; Bennett and Hairui, 2004; Bonfiglio et al., 1999; Capka and Carter, 2006). Indications of MEs are not limited to Ion suppression or enhancement, but retention time shifts due to deposition of phospholipids inside the analytical column, elevated baseline because of the presence of different phospholipids in the extracted samples, fluctuating calibration curves, (Bonfiglio et al., 1999; Brugger et al., 1997.) and inconsistent responses due to inconsistent ionization which are also attributed to the presence of ME in the applied analytical method.

Matuszewski (2006) had introduced the concept of relative matrix effect in terms of standard line slope. In the present manuscript, we have taken this concept a step forward. We have calculated the slope of different calibration curves by using the same as well as different lots of plasma. We have also compared the percent coefficient of variance (%CV) of slopes of the calibration curves prepared from same and different lots of plasma using three different types of internal standard (IS), one is the stable isotope labeled-IS (SIL-IS), another one is analogous to the analyte to be analyzed and last one has no similarity with analyte to be analyzed. We have also calculated the significance value (p-value) of each experiment to determine which approach is more significant over others, so that one can apply those methods to overcome matrix effect related issues during bio-analysis.

2. Experiment

2.1. Apparatus and software

The high performance liquid chromatography (HPLC) system with an auto sampler was a Shimadzu SIL-HTc (Shimadzu, Japan) and solvent delivery module was LC-10ADvp coupled with Applied Biosystem (MDS Sciex, ON, Canada) API 3000 or 4000 Tandem mass spectrometer as the detection system. Electro spray ionization source (ESI) was attached with the MS/MS system. All chromatographic integrations were performed by the Analyst software (version: 1.4.2; Applied Biosystems, ON, Canada). Hand vortexer used for protein precipitation was procured from Remi (Mumbai, India). Multi-pulse vortexer used for liquid–liquid extraction was purchased from Glas-Col (Cole Parmer, USA). The Caliper turbovap LV concentration workstation that was used to evaporate the samples was purchased from Caliper Life Sciences (Hopkinton, MA, USA). Positive pressure unit used for SPE was obtained from Orochem technologies Inc (Lombard, IL, USA).

2.2. Chemicals and reagents

Formic acid, ethyl acetate and methyl tertiary butyl ether (MTBE) were procured from Merck Specialties Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in our laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J. T. Baker, USA. Fresh frozen human plasma (K_2-EDTA as anticoagulant) was used during sample analysis, and was supplied by Clinical department of Cadila Pharmaceuticals Limited, Dholka, Ahmedabad, India. Plasma was stored into −70 ± 5 °C. Oasis HLB 30 mg, 1 cc cartridge was procured from Waters Corporation India (Ahmedabad, India) and Lichrosep, 30 mg, 1 mL cartridges were purchased from Merck India Limited (Mumbai, India).

2.3. Experimental design

In the first experiment, only one compound was taken into consideration. In this experiment calibration curves were prepared from 10 different plasma lots, among which six lots were normal plasma (Plasma Lot 1–6 in Table 1), two lots each of lipemic plasma (Plasma Lot 7–8 in Table 1) and hemolyzed plasma (Plasma Lot 9–10 in Table 1). Moreover, six calibration curves were prepared from each plasma lot. The %CV of the calibration standard slope values of individual lots of plasma was calculated. Two separate extraction techniques were used to prepare the calibration curve samples from each lot of plasma. The techniques were protein precipitation and solid phase extraction (SPE). In both the cases SIL-IS was used. We will call this experimental design as 'Experiment-1' in the rest of this manuscript.

In the second experiment, a more detailed investigation using 15 different analytes was performed. In this experiment, for each individual analyte six calibration curves from six different plasma lots by using three different IS were prepared. So, three sets of calibration curves for each analyte from each plasma lot were prepared. In one set stable isotope labeled-internal standard (SIL-IS) was used, whereas, in the second set the used IS was analogous to the compound under analysis, and in the last set, the used IS was from a completely different group than the actual compound of interest. In each case the %CV of calibration curve slope value for each analyte as well as the overall %CV of the calibration slope values of all analytes was examined. In this second experiment matrix factor (MF) was also calculated for each analyte to determine the degree of matrix effects. This experiment will be called as 'Experiment-2'.

In both the above experiments, all calculations were done twice. Initial calculation was performed, by applying the sample extraction method which showed the matrix effect, then by using different sample extraction method after removing the ME i.e. by changing the extraction techniques.

3. Results and discussion

3.1. Experiment-1

During this experiment calibration curve slopes were compared. Results are presented in Table 1. While analyzing the slope values, it was observed that higher slope values were obtained by protein precipitation technique samples, since these samples showed matrix effects (Fig. 1). All observed %CV of slope values were above 7, particularly the lipemic and hemolyzed plasma showed very high %CV of slope value, greater than 10.

On the other hand, when solid phase extracted samples were analyzed, then the observed %CV of slope values were within 4, which includes the lipemic and hemolyzed plasma samples too. The samples processed by SPE technique are free from any matrix effects which are clearly observed during qualitative experiment of matrix effect through post column infusion (Fig. 2) technique. Calibration curve slope values of all 10 plasma lots with their respective %CV values of before and after removing the matrix effect are presented in Table 1. All these methods were developed and validated in our laboratory during the past one year.

3.2. Experiment-2

In Experiment-2 some more detailed research work, by analyzing 15 different analytes was performed. Calibration curve slopes of each analyte were calculated by using six different plasma lots. Moreover, each analyte was analyzed by spiking three different IS separately. For each analyte 18 calibration curves are prepared. Hence, here total 270 slopes of different calibration curves were analyzed. In addition, the slopes values before and after removing the matrix effect were also analyzed. The average matrix factor value of all these analytes had a clear picture about the relation of matrix factor i.e. matrix effects with calibration curve slope values.

During the first phase of the analysis, all analytes were extracted from plasma samples by using different extraction techniques which included protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE). The matrix factor was calculated to determine the degree of matrix effects of all these extraction techniques by using SIL-IS only. Matrix factor was calculated by using any one among the six plasma lots used for each analyte. Six samples of middle QC concentration were prepared from that single plasma lot to calculate the average MF. Table 2, represents the summary of all these values. Column A represents the average matrix factor of each analyte, which shows approximately 40–70% ion suppression. Column B represents the %CV value of calibration curve slopes obtained from six different plasma lots using SIL-IS, all the slope values are within 10, except two values, which are 12.57 and 13.28 respectively. Column C contains the %CV value of calibration curve slopes. During analysis it was observed that all %CV values except the two mentioned in column B are within 15. In this experiment, IS used is analogous to the actual compound to be analyzed. Similarly, column D in Table 2 describes the %CV of slope values obtained by using the IS which belongs to a completely separate group compared to the main analyte. Here also, 13 samples showed that the %CV value was within 20, but two samples had the %CV of the slope value as 21.34 and 22.38 respectively.

In the second phase of the analysis, all those samples which were analyzed during the first phase were extracted by using different/modified extraction techniques to remove/ minimize the matrix effect. The obtained results are very interesting. The results are mentioned in Table 3. The MF value was calculated, which ranges from approximately 0.86–1.10. Column B represents the %CV value of the slopes of SIL-IS, which ranges from 1.15 to 3.51, except the 6th and 13th samples, whose %CV are close to 8, whereas, all other samples have %CV values within 4. In the C column, %CV values are within 5, except the same two samples, whose values are 7.54 and 7.89 respectively. Similarly, in the D column %CV values are within 3–5. But, the 6th and 13th samples have %CV values of 8.98 and 9.01.

Further, to investigate the significance of IS on matrix effect i.e. matrix factor, the ANOVA analysis was performed, where MF value and %CV of calibration curve slope values of different types of IS (e.g. SIL-IS, analogous IS and non-analogous IS) were taken into consideration to calculate the significant value (p-value). During the calculation of p-value two cases were considered. In first case, p-value was calculated by considering all the %CV values mentioned in Tables 2 and

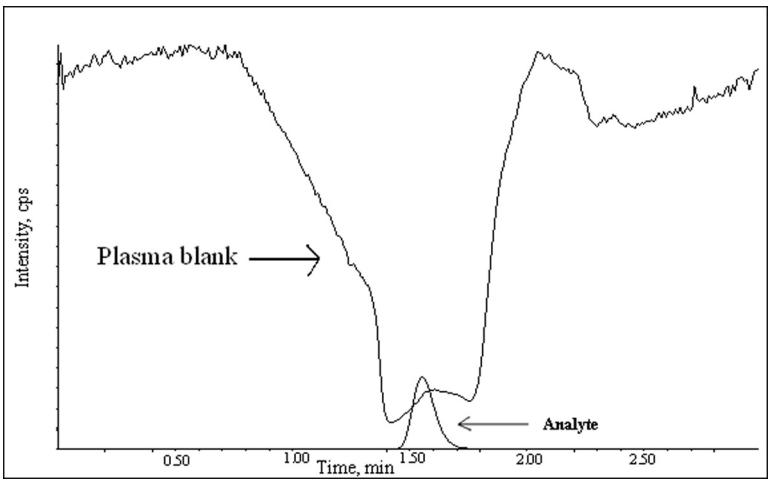

Figure 1 Post column infusion of methanol precipitated plasma blank sample.

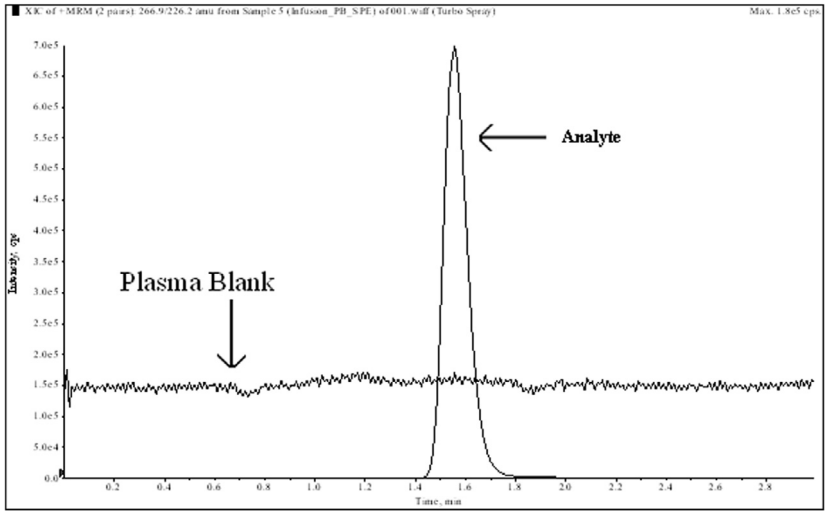

Figure 2 Post column infusion of plasma blank sample extracted through lichrosep sequence solid phase extraction cartridge.

3. Whereas, in the second case, *p*-value is calculated after removing the %CV value of 6th and 13th analyte.

Table 4 represents the summary of different *p*-values. It is observed that samples with SIL-IS show the most significant result, even samples having the matrix effects. Samples which are free from any matrix effect related issues and processed with analogous IS show $p < 0.05$. Sample having the matrix effects and processed with analogous IS, has the *p* value > 0.05. From the table it is also observed that if the calculation is performed after removing the slope value of 6th and 13th sample, *p* 0.05. Moreover, 6th and 13th sample showed abnormal slope values in all three cases, i.e. with SIL-IS, analogous IS and different IS. So, further investigations to find out such abnormal behavior of these samples were carried out.

As an investigation process, first the qualitative assessment of matrix effects was performed by post column infusion method. During this experiment it was observed that 6th sample showed matrix effects in the first half of the ion suppression region. Fig. 3, represents this fact very clearly. In this figure first half of the ion suppression region is marked as 'B'. Similarly, the analyte peak of the 13th sample comes in the second half of the ion suppression region, which is represented in Fig. 4. All other samples have their peaks within the ion suppression region (Fig. 5).

During method development of these 15 analytes some very interesting observations were noticed. The formation of phospholipids ions inside the mass rail was studied for which the total ion counts (TIC) were calculated. The role of different gas parameters like curtain gas (CUR), nebulizer gas (NEB) and collision gas (CAD) on formation of phospholipids ions and hence on TIC was monitored. The declustering potential (DP) impact on TIC was also studied. There are many other mass parameters which control the TIC, but for the simplicity of calculation, and ease of understanding only these four parameters have been monitored and presented in this manuscript.

During this study the following phospholipids at m/z $496 \rightarrow 184$ and $524 \rightarrow 184$ for lysophosphophatidylcholines, and m/z $704 \rightarrow 184$, $758 \rightarrow 184$, $786 \rightarrow 184$, $806 \rightarrow 184$ and $823.4 \rightarrow 184$ for glycerophosphocolines were monitored. Along this MRM transitions, one more MRM transition was added into it i.e. m/z $184 \rightarrow 184$, to check whether m/z 184

Table 1 Statistical results of single analyte in 10 different plasma lots.

Plasma lot (Sr. No.)	Before removing ME (protein precipitation)				After removing ME (SPE)			
	Slope (m)	Average	Standard deviation (SD)	% Coefficient of variance (%CV)	Slope (m)	Average	Standard deviation (SD)	% Coefficient of variance (%CV)
1	0.785826	0.792314	0.086042	10.86	0.812567	0.802124	0.01591	1.98
	0.891257				0.802543			
	0.692548				0.821576			
	0.725964				0.781249			
	0.865974				0.792684			
2	0.852678	0.757024	0.091863	12.13	0.812536	0.810647	0.015908	1.96
	0.812538				0.835486			
	0.613267				0.792584			
	0.732946				0.802576			
	0.773692				0.810052			
3	0.631258	0.742005	0.07889	10.63	0.736842	0.740495	0.018654	2.52
	0.751264				0.753624			
	0.702543				0.725413			
	0.835426				0.765241			
	0.789536				0.721356			
4	0.694587	0.763651	0.071741	9.39	0.808791	0.814446	0.01451	1.78
	0.865124				0.821536			
	0.785321				0.795236			
	0.778974				0.812543			
	0.694251				0.834125			
5	0.812567	0.770616	0.074695	9.69	0.759684	0.755311	0.019331	2.56
	0.721536				0.725694			
	0.832169				0.778695			
	0.663157				0.761234			
	0.823651				0.751248			
6	0.698694	0.786304	0.061221	7.79	0.802543	0.807351	0.009354	1.16
	0.795386				0.793658			
	0.823514				0.816984			
	0.756984				0.809876			
	0.856942				0.813694			
7	0.802367	0.671032	0.130681	19.47	0.753698	0.788131	0.026464	3.36
	0.506582				0.802694			
	0.689424				0.776954			
	0.569834				0.783694			
	0.786954				0.823615			
8	0.869458	0.733962	0.105936	14.43	0.813625	0.802251	0.009692	1.21
	0.713648				0.802543			
	0.805694				0.793624			
	0.598647				0.809876			
	0.682364				0.791586			
9	0.823648	0.73415	0.097596	13.29	0.823614	0.813516	0.015345	1.89
	0.702366				0.816547			
	0.642135				0.802543			
	0.851362				0.793624			
	0.651237				0.831254			
10	0.802354	0.736785	0.081092	11.01	0.812457	0.798835	0.010924	1.37
	0.651234				0.803625			
	0.725634				0.793215			
	0.836246				0.783624			
	0.668457				0.801254			

precursor ions get generated in the ion source. The general principles of phospholipid fragmentation were described by Brugger et al., who demonstrated that a positive-ion mode precursor-ion scan of m/z 184 is specific for phosphocholine-containing phospholipids, i.e., phosphatidylcholine and sphingomyelin. The lysophosphatidylcholine product-ion spectrum in the positive-ion mode displayed several ions originated from the collision-induced dissociation of the phosphocholine head group, which includes the most intense peak at m/z 184 and comparatively a less intense peak at m/z 104. Since lysophosphatidylcholine represents only 10% of the total plasma phospholipids, m/z 104 was not included into MRM scan transition.

The MRM transitions as mentioned in the preceding paragraph will detect only lysophosphophatidylcholines (lyso-PC), glycerophosphocolines (PC) and sphingomyelin (SM). This

Table 2 MF and Slope values of 15 different analytes calculated before removing ME.

Sample Sr. No.	Matrix factor (MF) [A]	%CV of slope values		
		SIL-IS [B]	Analogous IS [C]	Different IS [D]
1	0.5123	9.12	13.69	15.67
2	0.7026	7.78	11.28	14.05
3	0.3812	8.88	15.01	17.75
4	0.6473	9.24	14.44	16.68
5	0.5581	8.19	12.59	15.67
6*	**0.4914**	**13.28**	**17.59**	**21.34**
7	0.6827	8.56	11.25	14.57
8	0.4127	9.57	12.34	15.58
9	0.4826	8.19	11.58	14.51
10	0.5421	9.09	10.98	12.94
11	0.6657	7.27	10.57	13.24
12	0.5894	8.64	12.33	15.40
13*	**0.6357**	**12.57**	**16.67**	**22.38**
14	0.5128	8.28	11.59	17.31
15	0.4058	9.37	12.67	16.37

Table 3 MF and Slope values of 15 different analytes calculated after removing ME.

Sample Sr. No.	Matrix factor (MF) [A]	%CV of slope values		
		SIL-IS [B]	Analogous IS [C]	Different IS [D]
1	0.9215	2.14	2.98	4.54
2	0.8929	3.14	3.74	4.25
3	0.8615	3.51	2.2	3.98
4	0.9014	2.67	2.5	3.59
5	1.0104	2.01	4.01	4.55
6*	**0.9535**	**7.89**	**8.25**	**9.01**
7	0.8864	3.31	3.02	4.21
8	0.9237	1.99	2.67	4.28
9	0.9784	1.29	2.99	3.67
10	1.1024	1.15	3.32	3.02
11	1.0281	1.19	2.51	3.97
12	0.9684	2.02	3.98	4.58
13*	**0.9919**	**7.54**	**7.82**	**8.98**
14	0.9438	1.24	2.2	4.21
15	0.9784	2.53	3.36	3.67

Table 4 *p*-value obtained by ANOVA analysis.

Different situations	*p*-Value		
	SIL-IS	Analogous IS	Different IS
W_Aberrant_WME	0.5342	0.4255	0.5030
W_Aberrant_WOME	0.5077	0.9916	0.4819
WO_Aberrant_WME	0.0417	0.1372	0.0649
WO_Aberrant_WOME	0.0015	0.0085	0.2741

Note:
W_Aberrant_WME: slope value calculated with aberrant value with matrix effect.
W_Aberrant_WOME: slope value calculated with aberrant value but without matrix effect.
WO_Aberrant_WME: slope value calculated without aberrant value with matrix effect.
WO_Aberrant_WOME: slope value calculated without aberrant value but without matrix effect.

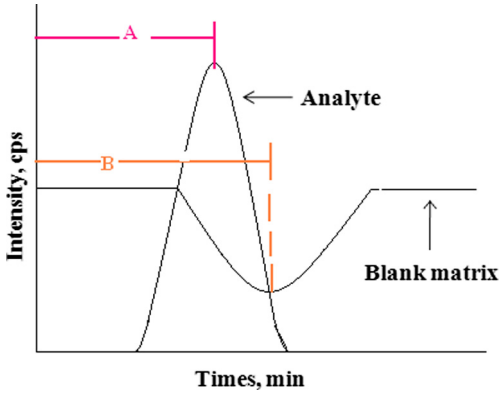

Figure 3 Matrix effect of 6th sample.

technique cannot be used to monitor the other phospholipids, such as phosphotidylethanilamine (PE), phosphotidylinositol (PI), phosphotidylglycerol (PG), phosphotidylserine (PS) and phosphatidic acid (PA), since these phospholipids do not generate the *m*/*z* 184 ion in the source. Consequently, to identify any phospholipids other than the above mentioned MRM

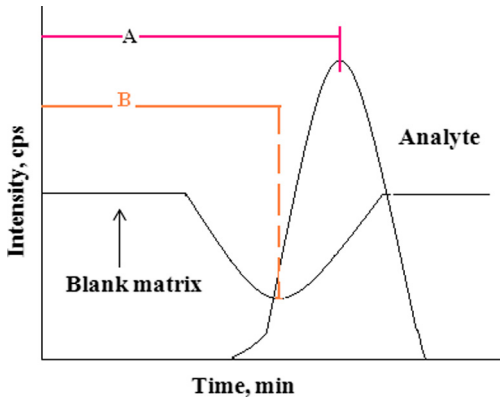

Figure 4 Matrix effect of 13th sample.

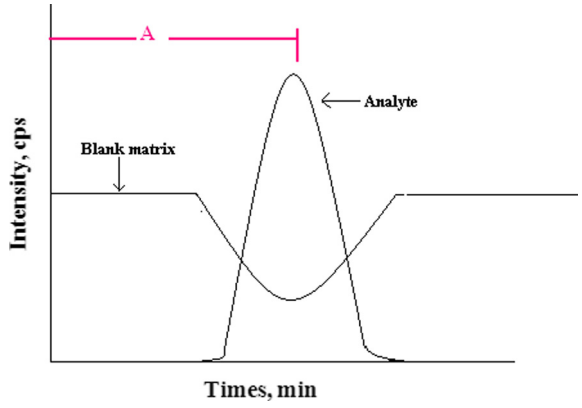

Figure 5 Ion suppression of all other samples (except 6th & 13th sample).

transitions, precursor ion scan at m/z 104 and m/z 184 was performed in positive polarity in ESI interface.

While studying with CAD gas, it was observed that TIC was higher in lower CAD value. At lower CAD values parent ions did not get fragmented and reached to the detector as it is, resulting in a higher TIC value. When CAD values increased, parent molecules of phospholipids got fragmented to their daughter ions having the common m/z 104.0 and 184.0. The significance test through ANOVA was conducted, which generated the $p < 0.05$. The changes of TIC on varied CAD gas

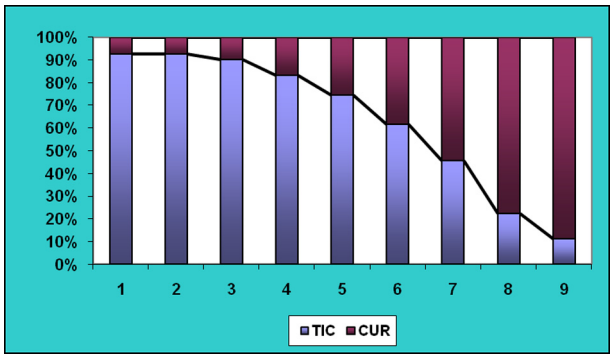

Figure 7 TIC of phospholipids with different CUR gas values.

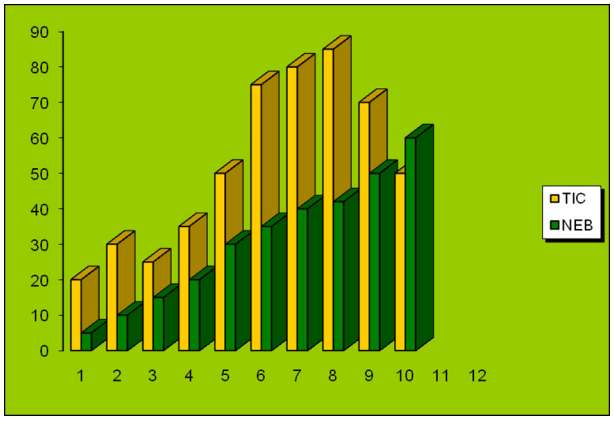

Figure 8 TIC of phospholipids with different NEB gas values.

are presented in Fig. 6. The significance value of TIC with varying degrees of CUR gas was calculated. The obtained p value is <0.05, hence significant. Fig. 7 depicts the relationship between TIC and CUR gas. Moreover the higher value of CUR gas leads to in-source fragmentation (ISF) of phospholipids ions, which also contribute to the TIC. Similarly, the role of nebulizer gas on TIC of phospholipids was studied. It was observed that it does not follow any pattern like CUR and CAD gas. The obtained p value is <0.05 by ANOVA. Fig. 8, represents the graph of TIC vs. NEB gas. The variation of phospholipids ionization with DP is also mentioned through Figs. 9 and 10 represents the overall change in TIC with the variation of the above mentioned four parameters.

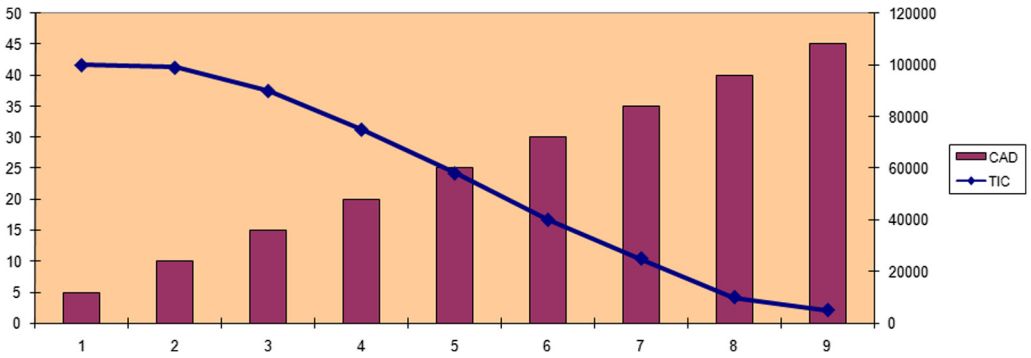

Figure 6 TIC of phospholipids with different CAD values.

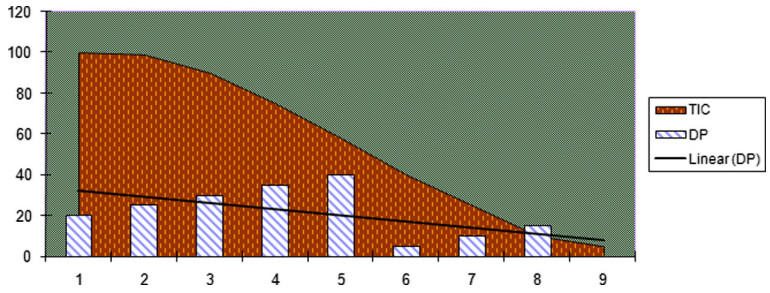

Figure 9 TIC of phospholipids with different DP values.

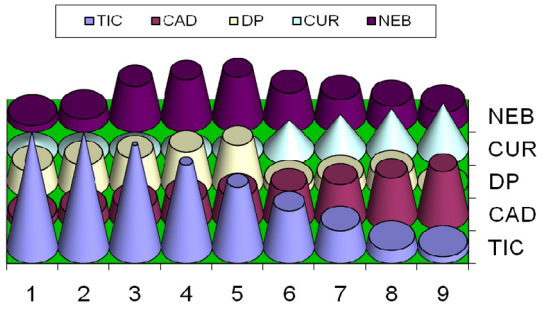

Figure 10 Change in TIC with different mass parameters.

4. Conclusion

In the present manuscript, the calibration curve slopes obtained from different plasma lots have been compared to study the relative matrix effect. The higher %CV of slope values were observed when the extracted samples showed ME, but after removing the ME the %CV values of the slopes decreased to normal range. During the experiment it is also observed that SIL-IS gives the best results even with those sample extraction techniques where ME were not removed. But, after removing the ME one can use analogous IS for routine sample analysis, which is a cost effective approach. Moreover, in 'Experiment-2', where 15 analytes were studied, it is observed that if the %CV of the calibration curve slope is within 5, then we can assume that the method is less affected with matrix effect related issues, though it is always recommended to perform the qualitative and quantitative determination of ME during the method validation stage.

In 'Experiment-2', for 6th and 13th analyte, the abnormalities with respect to obtained %CV value were observed. It is a good learning for all bio-analytical scientists that during method development they should take sufficient care of the chromatographic parameters, so that the analyte/IS peak did not fall in the upward or downward slope region of the matrix effects area, where almost 50% of the peak falls in the ion suppression region and the remaining portion in the no-matrix effect region. Ion generation is highly inconsistent in these two regions. Even, SIL-IS cannot compensate this inconsistent ionization. So, the %CV of the calibration curve slope value was close to 13 for these two samples. Though the chromatographic peaks of all other remaining samples were within the ion suppression region, which showed comparatively better %CV value, these were not acceptable and a modification of the analytical technique was required.

From all the performed experiments it is observed that determination of relative matrix effect by comparing the calibration standard slope value is very essential, particularly when sample size for pharmacokinetic study is large i.e. $n > 50$ because, in actual study sample analysis the matrix lot used to prepare the calibration curve (CC) samples will be different from the subject sample matrix. Even, multiple subjects can be analyzed by using a single CC. If the applied analytical method is not free from ME, then same subject sample will give different concentration values with different CC standards prepared from different plasma lots. These variations in obtained concentration are because of high variation in the obtained slope values from these different calibration curves. This will raise the question on the reliability of the results obtained by using this analytical method. So, it is recommended to perform the "relative" matrix effect experiment during bio-analytical method validation. To perform this experiment at least three precision and accuracy batches from three different plasma lots should be prepared. Then calculate the %CV of the three calibration standard slope values. If %CV value is more than 5, then the applied analytical method should not be used for actual pharmacokinetic study sample analysis and the analytical method should be modified/re-developed.

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